homocysteinyl]-3'-deoxy-(S)-glycerol; 2',3'-acyclic 8-aza-SAH (6), 2'-[O-[(R)-homocysteinyl(8-azaadenin-9-yl)methyl]]-3'-[S-(R)-homocysteinyl]-3'-deoxy-(S)-glycerol; HMT, histamine N-methyltransferase; COMT, catechol O-methyltransferase; PNMT, phenylethanolamine N-methyltransferase; HIOMT, hydroxyindole O-methyltransferase.
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Inhibition of Beef-Brain and Dog-Heart ($Na^+ + K^+$) Activated Adenosine Triphosphatase by Carbon-3 Branched Cardenolides

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Twenty-two C-3 branched cardenolides were investigated as inhibitors of beef-brain and dog-heart ($Na^+ + K^+$) activated adenosine triphosphatase. The synthetic compounds had lower inhibitory strength than digitoxigenin, and there was no indication of an improved safety index. Structure–activity relationships show that increased steric shielding of the 3-OH group results in reduced inhibition.

During our continued interest in the design and synthesis of cardiac glycosides with improved safety indices, a series of C-3 branched cardenolides was synthesized.[1-3] It was then of interest to evaluate if there exists any structure–activity relationship for these compounds.

Following the first report by Schatzmann[4] that ouabain binds to the ($Na^+ + K^+$) ATPase enzyme responsible for the active transport of potassium and sodium, a large amount of evidence was gathered which showed that the mode of action of cardiac glycosides is their inhibition of a membrane or microsomal ($Na^+ + K^+$) activated adenosine triphosphatase (E.C. 3.6.1.3).[5] We first selected beef-brain microsomal ATPase for our investigations, since it was reported that the receptor site of ATPase for cardiac glycosides is the same in all organs of the same species.[6,7] Thereafter, microsomal ATPase from dog hearts was also used.

Experimental Section

Beef brains were obtained fresh from the municipal slaughterhouse, Mannheim, and dog hearts were kindly supplied by Dr. Engelmann from our pharmacology department. The organs were frozen immediately and only partially thawed prior to use. Microsomal ATPase was isolated from beef brains according to Klodas et al.,[8] while for the isolation from dog hearts a slightly different procedure was used.[9] In accordance with Schwartz et al.,[10] the enzymatic activity of ATPase was followed in a linked enzyme system by reaction of primarily liberated ADP with pyruvate kinase and subsequent reaction of the pyruvate with NADH in the presence of lactate dehydrogenase. The monitoring of the oxidation of NADH in the photometer is a direct measure for the activity of ATPase. In the case of the inhibition of beef-brain ATPase, the linked enzyme system was incubated with the inhibitor for 5 min prior to addition of the substrate (ATP). The inhibitor was either dissolved in 1.0 mL of water or first in a few drops of dimethyl sulfoxide which was then made-up with water to a final volume of 1.0 mL. Under these conditions dimethyl sulfoxide did not effect the enzymatic reaction. The final volume of the reaction mixture was 2.95 mL. For inhibition studies with dog-heart ATPase, the microsomal enzyme preparation in a magnesium chloride, potassium chloride, and sodium chloride containing Tris–HCl solution (pH 7.4) was incubated with inhibitor solution for 5 min, after which time were added pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate, and NADH. The reaction was then started by adding ATP.

The enzymes, substrates, and reagents were obtained from Boehringer Mannheim and were of analytical grade. The photometer used was an Eppendorff Model 1101 equipped with an automatic cuvette programmer (Model 2702) and a Model 4412 recorder.

The inhibition of ATPase by the C-3 branched cardenolides was determined at at least four different inhibitor concentrations which were about equidistant on the log scale, and each determination was run in duplicate or triplicate. The linear regression of the percent inhibition and the logarithm of the inhibitor concentration were calculated on a Hewlett-Packard Calculator 9100 B.

Results

The isolation of microsomal ($Na^+ + K^+$) activated ATPase from beef brain resulted in preparations with enzymatic activities of about 50 μmol of ATP used per milligram of protein per hour. More than 95% of this activity was sensitive to ouabain or digitoxigenin inhibition. The enzymatic reaction was linear for at least 20 min. The ID_{50} values of the inhibitors with their confidence limits in parentheses are given in Table I. In Figure 1 the

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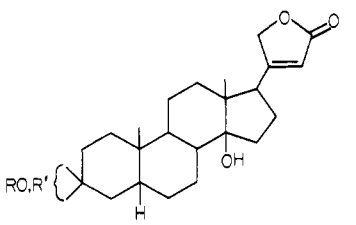
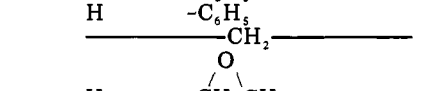
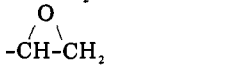
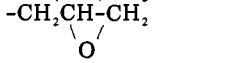
Table I. Inhibition (ID_{50})[a] for Beef-Brain ATPase



R	R'	no.	ID_{50}, μmol, 3-β-OR	no.	ID_{50}, μmol, 3-α-OR
H	-H	1	0.096 (0.087-0.107)	2	1.17 (1.06-1.28)
H	-C≡CH	3	0.25 (0.23-0.27)	4	2.11 (1.79-2.45)
H	$-CH=CH_2$	5	0.31 (0.29-0.33)	6	6.68 (5.90-7.57)
H	$-CH_3$	7	0.92 (0.75-1.13)	8	11.37 (10.95-11.83)
H	$-CH_2CH=CH_2$	9	1.25 (1.17-1.35)	10	5.81 (5.41-6.24)
H	$-C_2H_5$	11	2.15 (1.87-2.48)	12	6.40 (5.31-7.71)
H	$-C_6H_5$	13	2.11 (1.86-2.41)	14	5.81 (5.32-6.34)
		15	0.71 (0.63-0.80)	16	0.74 (0.70-0.80)
H		17	2.03 (1.94-2.13)	18	3.17 (3.03-3.30)
H	$-CH(OH)CH_2OH$	19	15.37 (14.92-15.83)	20	1.70 (1.64-1.77)
H		21	3.65 (3.36-3.97)	22	4.06 (3.89-4.23)
H	$-CH_2CH(OH)CH_2OH$	23	7.66 (7.13-8.33)	24	2.34 (2.25-2.43)

[a] Confidence limits given in parentheses.

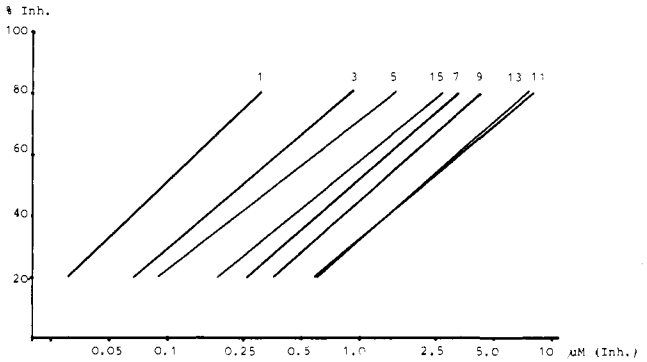


Figure 1. Inhibition of beef-brain ATPase by C-3 branched digitoxigenins.

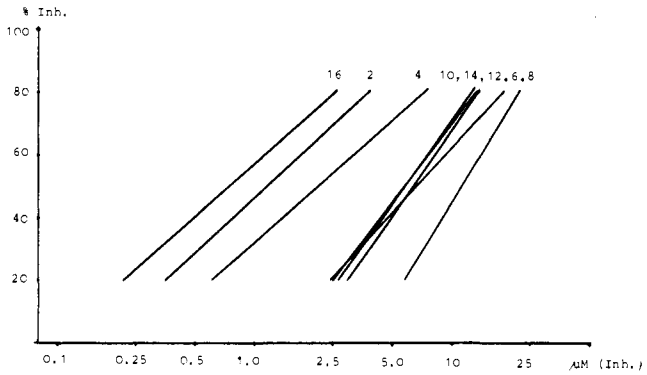


Figure 2. Inhibition of beef-brain ATPase by C-3 branched epidigitoxigenins.

relationship between inhibitor concentration and percent inhibition for C-3 branched alkyl- and aryl-substituted cardenolides is presented. As in all of the following figures, the regression lines have coefficients of correlation of 0.97 or better. Compounds given in Figure 1 show a nearly parallel slope, but all C-3 branched derivatives show smaller inhibition than digitoxigenin (**1**).

Figure 2 illustrates the dependence for epidigitoxigenin (**2**) (epidigitoxigenin naming the compound having the 3-hydroxyl group in the α position as opposed to digitoxigenin) and its derivatives. Here only the stronger

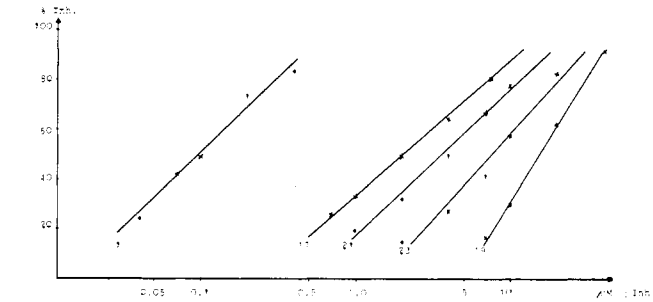


Figure 3. Inhibition of beef-brain ATPase by C-3 branched digitoxigenins with oxygen functions in the side chain.

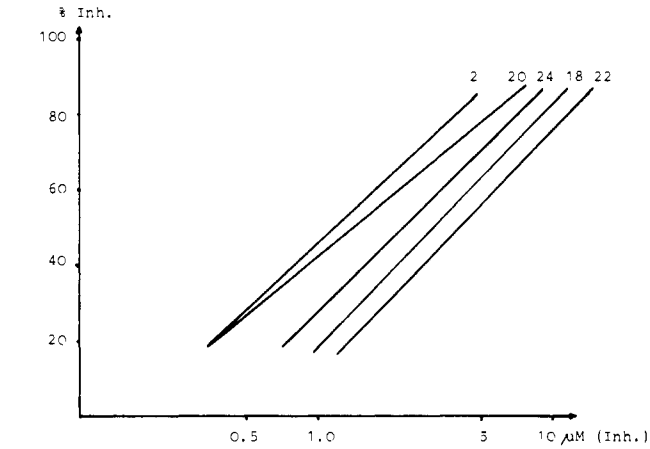


Figure 4. Inhibition of beef-brain ATPase by C-3 branched epidigitoxigenins with oxygen functions in the side chain.

inhibitors have a parallel slope, with all the derivatives having a lower strength of inhibition.

The inhibition of beef-brain ATPase by C-3 branched digitoxigenins with oxygen functions in the side chain is shown in Figure 3, while the corresponding epidigitoxigenin derivatives are shown in Figure 4. With the exception of compound **20**, the derivatives have a slope nearly parallel to the corresponding unsubstituted compounds. The deviation from parallelism increases with decreasing inhibitory strength.

Table II. ID_{50}[a] Values for Dog-Heart ATPase

no.	ID_{50}, μmol, 3-β-OR	no.	ID_{50}, μmol, 3-β-OR
1	0.035 (0.032-0.037)	13	0.60 (0.57-0.63)
3	0.12 (0.11-0.13)	15	0.275 (0.26-0.29)
5	0.092 (0.087-0.098)	17	1.10 (1.03-1.18)
7	0.32 (0.29-0.36)	19	7.80 (7.06-8.62)
9	0.55 (0.50-0.61)	21	1.69 (1.60-1.79)
11	0.73 (0.68-0.78)	23	4.44 (4.17-4.74)

[a] Confidence limits given in parentheses.

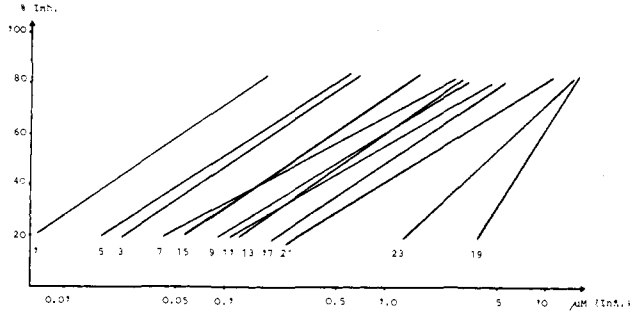


Figure 5. Inhibition of dog-heart ATPase by C-3 branched cardenolides.

The microsomal preparation of $(Na^+ + K^+)$ activated ATPase from dog heart had only one-fourth the activity of that from beef brain. Therefore, only the derivatives of digitoxigenin were selected for this system. The corresponding ID_{50} values for the inhibitors are given in Table II. The relationships between inhibitor concentration and percent inhibition are given in Figure 5. The parallelism of the regression lines is not as well expressed as in the case of the beef-brain system.

Discussion

The C-3 branched derivatives of digitoxigenin have lower inhibitory activity than the parent compound. From the nearly parallel slope of the regression lines (Figures 1 and 3) up to an inhibitor concentration of about 5 μM, a concentration nearly 100 times that for inhibition of ATPase by digitoxigenin, an identical mode of action of these compounds on the $(Na^+ + K^+)$ activated ATPase can be assumed. As can be seen from Figure 1, increased steric shielding of the 3-OH group of digitoxigenin by C-3 substituents causes a loss of inhibitory strength. This observation underlines the importance of the 3-OH group of digitoxigenin for inhibition of ATPase. Unfortunately, too little is known about the binding site for inhibitors of ATPase for another explanation of the observed data to be excluded, namely, that the reduction in activity could be caused in part by steric hindrance of the close association of the steroid ring system with the enzyme receptor site.

Inhibition of $(Na^+ + K^+)$ activated ATPase between 20 and 50% is necessary for therapeutic action (positive inotropic), while above 50% inhibition toxic effects of the cardiac glycosides become evident.[6,7] A decrease in the slope of the regression lines would, therefore, indicate an improved safety index. The parallelism of the regression lines of the C-3 branched compounds with that of digitoxigenin shows that these compounds do not have an improved safety index.

In the series of the C-3 branched epidigitoxigenins (Figures 2 and 4), again a parallelism of the slopes of the regression lines can be observed. With the exception of compound **16** [spiro(oxirane-3,3′-cardenolide)], all compounds have lower inhibitory activity than epidigitoxigenin, which in itself is about 10 times weaker an inhibitor than digitoxigenin. The steric shielding of the 3-OH group is not as clear-cut in this series as it is for the digitoxigenin derivatives.

An interesting observation can be obtained by comparing those cardenolides that bear oxygen functions in the C-3 side chain. In the digitoxigenin derivatives the opening of the epoxide ring to the dihydroxy compounds (**17** vs. **19** and **21** vs. **23**) leads to a considerable decrease in inhibition, while in the epidigitoxigenin derivatives about a twofold increase in inhibition is seen (**18** vs. **20** and **22** vs. **24**). The dihydroxy compounds derived from epidigitoxigenin are much stronger inhibitors than their corresponding digitoxigenins. While digitoxigenin derivatives fit into previously stated findings which show that increased steric shielding of the 3-OH group leads to decreased inhibition, it seems that in the epidigitoxigenin derivatives a hydroxyl group of the C-3 side chain mimics the action of the 3-hydroxyl group and thus leads to better inhibitors.

Epimeric spiro(oxirane-3,3′-cardenolides) **15** and **16** have nearly identical ID_{50} values and slopes of the regression lines. Besides that, compound **16**, with oxygen in the α position, even shows improved inhibitory activity over the corresponding epidigitoxigenin (**2**). These observations can be explained by the fact that the spirooxiranyl ring forces the C-3 oxygen into an intermediate position between α and β, as can be seen from stereochemical models.

In the dog-heart system nearly identical results were obtained, but these are not as clear-cut. Again we find increased steric shielding of the 3-OH group with increased bulkiness of the C-3 branch (Figure 5). Futher investigations into structure–activity relationships of C-3 branched cardenolides are in progress.

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